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Extracellular matrix regulates apoptosis in human neutrophils

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Background. During inflammation, polymorphonuclear neutrophils (PMNs) migrate into the affected tissue interacting with extracellular matrix (ECM) proteins. We tested the hypothesis that PMN–matrix interaction affects PMN apoptosis.

Methods. Apoptosis of human PMNs was detected by DNA-fragmentation assay and was quantitated by flow cytometry, ultraviolet and light microscopy. Cell adhesion was assessed by a toluidine blue assay, and cell spreading was detected by phase contrast microscopy. Protein tyrosine phosphorylation was studied using Western blotting and confocal microscopy.

Results. PMN apoptosis was not different in unstimulated cultures on either surface-adherent fibronectin or on PolyHema, a surface that prevents cell adherence. However, tumor necrosis factor-α (TNFα) treatment significantly increased apoptosis on fibronectin (37 ± 4%) compared with PolyHema (20 ± 3%). Tests on other matrix substances revealed that the percentage of apoptotic PMNs in the presence of TNFα was 8 ± 1% on PolyHema, 26 ± 4% on fibronectin, 17 ± 2% on collagen I, 16 ± 2% on collagen IV, and 16 ± 3% on laminin ($P < 0.05$ for all matrices compared with PolyHema). Preincubation with genistein (50 μM) significantly inhibited TNFα-mediated apoptosis on fibronectin (39 ± 4% to 21 ± 4%) but not on PolyHema (21 ± 4% to 16 ± 4%). Genistein also reduced PMN spreading on fibronectin. In contrast, inhibitors of mitogen-activated protein kinase and protein kinase C showed no effect on PMN apoptosis. Fibronectin strongly increased tyrosine phosphorylation of three 102, 63, and 54 kDa proteins. Five newly tyrosine-phosphorylated 185, 85, 66, 56, and 42 kDa bands were also visible. Using confocal microscopy, highest tyrosine phosphorylation was localized to sites of cell–matrix interaction.

Conclusions. ECM influences apoptosis in TNFα-activated, adherent, spreading PMNs. The process is regulated by tyrosine phosphorylation. Acceleration of apoptosis may shorten the PMN lifespan and thereby locally regulate inflammation.

Key words: inflammation, human neutrophils, tumor necrosis factor α, genistein, polymorphonuclear neutrophils, cell death, tyrosine phosphorylation.

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During inflammation, polymorphonuclear neutrophils (PMNs) leave the circulation and migrate into affected tissue. PMNs are dominant in early inflammatory infiltrates of experimental glomerulonephritis and are detected in several forms of human glomerulonephritis [1–3]. At the inflammatory site, a cytokine-rich environment causes PMNs to generate reactive oxygen species, release proteolytic enzymes, and respond by phagocytosis. Adherence to extracellular matrix (ECM) proteins or to other cells accelerates the PMN response. Adhesion-dependent activation in tumor necrosis factor-α (TNFα)-treated PMNs has been demonstrated for respiratory burst, degranulation, and phagocytosis [4–7]. Furthermore, engagement of β_2 integrins increases the TNFα-stimulated PMN respiratory burst on certain matrix substances [4, 8]. Recently, apoptosis was shown to be important for the resolution of inflammation. Apoptotic PMNs are recognized and removed by phagocytes before they disintegrate and release proteolytic constituents [9, 10]. It has been demonstrated in a rat model of immune complex glomerulonephritis that apoptosis is one of the mechanisms resulting in resolution of neutrophilic infiltration [1]. PMN apoptosis is regulated by a variety of cytokines that can either delay [11–15] or accelerate [16–19] PMN apoptosis. We and others have shown previously that TNFα treatment rapidly leads to apoptotic PMN cell death [16, 18, 19]. We now tested the hypothesis that PMN–matrix interaction affects PMN apoptosis, and found that TNFα-mediated apoptosis was significantly accelerated on fibronectin-coated surfaces. The effect was diminished by inhibitors of tyrosine phosphorylation but was not affected by protein kinase C (PKC) inhibitors or by mitogen-activating protein (MAP) kinase inhibitors. We identified five newly tyrosine-phosphorylated protein bands approximately 185, 85, 66, 56, and 42 kDa in size. Finally, spots of highest tyrosine phosphorylation were localized at adhesion sites between PMNs and the fibronectin-coated surface. Our findings demonstrate that ECM proteins accelerate TNFα-mediated neutrophil apoptosis *in vitro*.

This mechanism may be important for regulating the lifespan of PMNs actively engaged in inflammation.

MATERIALS

Reagents

The following reagents were purchased from Sigma (Deisenhofen, Germany): propidium iodide (PI), modified Wright Giemsa, 4′,6 diamidino-2-phenylindole (DAPI), ficoll-hypaque, bovine serum albumin (BSA), DNase-free RNase, poly-hydroxyl-ethyl-meth-acrylate (PolyHema), poly-L-lysine, collagen I and IV, laminin from human placenta, toluidine blue. Fibronectin from human plasma was obtained from Boehringer Mannheim (Mannheim, Germany). Genistein, SB 202190, PD 98059, staurosporin, and calphostin C were purchased from Calbiochem (Bad Soden, Germany). RPMI 1640, trypan blue, and phosphate-buffered saline (PBS) were obtained from Seromed (Berlin, Germany). Plasmagel was obtained from Cellular Products Inc. (Buffalo, NY, USA). Recombinant TNFα was obtained from Genzyme (Rüsselsheim, Germany). The phosphotyrosine antibody PY20 was acquired from Transduction Laboratories (Lexington, KY, USA), 1G2 from Boehringer Mannheim, and horseradish peroxidase-labeled goat antimouse from Pierce (Munich, Germany).

Human PMN isolation, cell count determination, and cell viability

Polymorphonuclear neutrophils were isolated from heparinized whole blood from healthy donors. The protocol included red blood cell sedimentation by Plasmagel followed by Ficoll-Hypaque density gradient centrifugation and hypotonic red cell lysis. Cells were resuspended at 10^7 per ml in RPMI 1640 supplemented with 2 mM glutamine and penicillin/streptomycin. Trypan blue exclusion was used to determine cell viability. PMNs 10 μl in suspension were incubated with 40 μl trypan blue for five minutes at room temperature (RT). Cells were counted in duplicate using a hemocytometer and were considered viable if able to exclude trypan blue.

Preparation of surfaces

Twelve-well tissue culture grade polystyrene plates were employed in studies on apoptosis and cell spreading, whereas adhesion assays were done in 96-well polystyrene plates. To achieve nonadherent conditions, wells were coated with 500 μl of PolyHema (50 mg/ml) as described elsewhere [20]. The following agents were used to achieve adherent conditions: fibronectin (10 μg/cm^2), laminin (2 μg/cm^2), collagen I (10 μg/cm^2), collagen IV (10 μg/cm^2), and poly-L-lysine (10 μg/cm^2). Coating was performed as suggested by the manufacturer.

Culture conditions of human polymorphonuclear neutrophils

Polymorphonuclear neutrophils 250 μl at 10^7/ml RPMI without fetal calf serum were pipetted into 12-well tissue culture plates (TPP-Company, Munich, Germany) that were coated as described earlier here. Two hundred forty-five μl of supplemented RPMI 1640 containing 20% heat-inactivated fetal calf serum were added followed by 5 μl of TNFα (20 ng/ml) or an equal volume of carrier protein containing PBS solution (0.5% BSA). The final cell concentration was 5×10^6 cells per ml. When indicated, cells were preincubated with 5 μl of a signal transduction inhibitor (or diluent control) for 15 minutes on ice prior to the stimulation with TNFα. Samples were incubated at 37°C in 5% CO_2. After the indicated times, cells were collected from PolyHema by pipetting and from all other dishes by scraping. In some experiments, cells were stained either with Wright Giemsa or with DAPI directly on the dish. All experiments were carried out in duplicate.

Apoptosis measurements with propidium iodide-stained PMNs

Flow cytometry was used to measure DNA content at the single-cell level as described previously [16]. The method takes advantage of the fact that activated endonucleases generate low molecular weight DNA fragments in apoptotic cells. After membrane permeabilization, these fragments leak out, resulting in decreased DNA content in apoptotic cells, whereas DNA content in nonapoptotic cells remains unchanged. Briefly, freshly isolated or cultured cells were spun at 200 g for five minutes at 4°C and were carefully resuspended in PBS containing 0.5 mM ethylenediamenetetraacetic acid (EDTA). Chilled 95% ethanol was added to a final concentration of 70%, and the cell mixtures were stored at −20°C for one to two days. PMNs were pelleted (200 g, 5 min at 4°C) and resuspended in 250 μl PBS/0.5 mM EDTA/1% BSA. Two hundred fifty μl PBS containing 200 μg DNase-free RNase and 500 μl PBS containing 50 μg propidium iodide (PI) were added. Cells were held for 15 minutes in the dark at room temperature and were then stored at 4°C for six to eight hours in the staining mixture, allowing low molecular DNA fragments to leave permeabilized cells. PMNs were analyzed using a fluorescent antibody cell sorter (FACscan; Becton Dickinson, Heidelberg, Germany), and 10,000 events per sample were collected in listmode using Lysis II software for data acquisition and analysis.

Cell staining with Wright Giemsa or DAPI and sulforhodamin 101

Cells were stained using either modified Wright Giemsa stain or a solution containing 1 μg/ml 4′,6 Dia-

midino-2-Phenylindole (DAPI). Morphologic features of apoptosis, such as pyknotic nuclei, nuclear and cytoplasmic condensation, and the formation of apoptotic bodies were assessed by microscopy.

DNA fragmentation assay

DNA fragmentation was studied using isolated low molecular weight DNA. Five $\times$ 10^6 PMNs were incubated in lysis buffer (0.5 mM Tris-HCl, pH 7.5, 20 mM EDTA, pH 8.0, 0.5% Triton X-100) for 30 minutes on ice. Lysate was centrifuged at 20,000 *g* for 20 minutes at 4°C to select for low molecular weight DNA. Supernatants were extracted twice with an equal volume of PCIA (Tris-saturated phenol:chloroform:isoamyl alcohol at 25:24:1) and twice with CIA (chloroform:isoamyl alcohol at 24:1). DNA was precipitated at −20°C overnight by adding 2.2 volumes of absolute ethanol in the presence of 0.3 M sodium acetate, pH 5.2. After centrifugation at 13,500 *g* for 20 minutes at 4°C, the DNA pellet was air dried and resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8) containing RNase Plus (5′-3′). After incubation for 30 minutes at 37°C, DNA was quantitated, and 7.5 μg were electrophoresed in a 1.5% agarose gel containing 0.5 μg/ml ethidium bromide, visualized under ultraviolet light, and photographed.

Western blotting

PMNs were cultured on either PolyHema or fibronectin in the absence or in the presence of TNFα (40 ng/ml) as described earlier here. Samples were harvested at the indicated time points, and cell lysates were prepared by resuspending 5 $\times$ 10^6 cells in 50 μl of ice-cold lysing solution (20 mM Tris-HCl, pH 8.0, containing 138 mM NaCl, 1% Triton X-100, 2 mM EDTA, 10% glycerol, 0.2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 0.1 mM quercetin, 5 mM iodoacetamide). Samples were stored for five minutes on ice and were centrifuged at 12,000 g for five minutes at 4°C. Supernatant was recovered, and protein concentration was estimated by bicinchoninic acid protein assay (Pierce, Rockford, IL, USA). Samples were incubated for five minutes at 95°C in loading buffer (250 mM Tris-HCl, pH 6.8, with 4% sodium dodecyl sulfate (SDS), 20% glycerol, 0.01% bromophenol blue), and 50 μg protein per lane were loaded on 9% SDS-polyacrylamide gel, electrophoresed, and blotted onto polyvinylidene difluoride membrane by semidry technique. Membrane was blocked in 1% BSA/0.05% Tween/PBS overnight at 4°C. Phosphotyrosine was detected using the monoclonal antibody PY20 (1:2,000 dilution) and a horseradish peroxidase-labeled goat antimouse IgG (1:1,000). Blot was developed by incubation in a chemiluminescence substrate (NEN Life Science Products) and was exposed to an x-ray film.

Immunocytochemistry

The techniques we used for confocal microscopy are described in detail elsewhere [21]. To analyze the effects of fibronectin on tyrosine phosphorylation in TNFα-treated PMNs, unbound cells were removed by washing, and adherent cells were fixed with 4% paraformaldehyde and were permeabilized with 80% methanol at −20°C. TNFα-treated PMNs that were cultured on PolyHema did not adhere. These cells were collected by pipetting followed by fixation and permeabilization as described earlier here. After incubation with BSA 1% in phosphate buffer solution (BSA/PBS) for 60 minutes, the preparation was incubated for one hour at RT with antiphosphotyrosine antibodies (clone 1G2; Boehringer Mannheim) diluted in PBS with 0.1% BSA (1:80), washed three times with PBS, and then exposed to the secondary antibody (FITC-conjugated antimouse IgG, at 1:100, 1% BSA/PBS; Dianova, Hamburg, Germany) for 60 minutes. Control stainings in which the primary antibody was omitted were performed in parallel. The preparation was mounted with 50% glycerol under a glass coverslip on a Nikon-Diaphot (Tokyo, Japan) microscope. A Biorad MRC 600 confocal imaging system (Bio-Rad Laboratories, Freiburg, Germany) with an argon-krypton laser (488/568 nm wavelength) was used. Subcellular localization of phosphorylated tyrosine was carried out with a pinhole setting of the confocal aperture resulting in a z-resolution of approximately 0.5 mm. Z-projections were calculated with the som/thruView software (Bio-Rad).

At least 30 cells from each of the three experiments were examined under each experimental condition. The reproducibility of the results was verified by two separate investigators. Quantitation of the signal intensity was done with histogram/area functions in the MRC-Comos software. The cells were outlined manually, and the calculated mean fluorescent intensity was obtained. Data are presented as the ratio of the mean fluorescent intensity in the respective regions to the mean fluorescent intensity of the whole cell area.

Assessment of adhesion and spreading of PMNs

Ninety-six–well plates coated with PolyHema, fibronectin, or poly-L-lysine, respectively, were used for the adhesion assay (coating as described earlier here). One $\times$ 10^5 PMNs in 100 μl medium were either left untreated or were treated with 20 ng/ml of TNFα with and without preincubation in genistein (50 μM) as described earlier here. Plates were incubated at 37°C in 5% CO_2 for the indicated time period. Wells were flicked dry and were washed three times with PBS, and adherent cells were fixed in 100 μl of 10% formalin for 15 minutes at RT. Plates were stained overnight with 100 μl of toluidine blue in 10% formalin. Plates were washed three times

with copious amounts of distilled water before lysis in 2% SDS for 10 minutes. Optical density (OD) was read at 600 nm with a microtiter plate reader. Each experiment was done in triplicate. Experiments confirmed the relationship between cell number and OD by construction of a standard curve with OD measured for increasing amounts of cells ($N = 5$ for each cell concentration from 1×10^4 to 1×10^5). The r^2 value for the standard curve was 0983, indicating an excellent correlation between OD and cell number over the range tested. Cells on PolyHema were assessed by phase contrast microscopy because the assay interfered with PolyHema. For estimation of cell spreading, PMNs were cultured in 12-well plates as described earlier here. At the indicated time points, nonadherent cells were discarded, and the percentage of spread cells was assessed. Each sample was counted by two investigators using phase contrast microscopy. At least 100 cells were counted, and those that were phase dark, enlarged, and irregular were considered to be spread.

Statistical analysis

Statistical analysis was performed on a Macintosh computer (Apple Inc., Cupertino, CA, USA) equipped with commercially available program (Statview; Cricket Software Inc., Philadelphia, PA, USA). Student's paired *t*-tests were performed to compare results between paired groups. For multiple comparisons repeated measures analysis of variance was used (SPSS, Chicago, IL, USA). Data are presented as mean ± SEM. Differences were considered to be significant when the *P* value was less than 0.05.

RESULTS

We first characterized the effect of TNFα on PMN adherence by an assay employing toluidine blue ($N = 7$). TNFα treatment (20 ng/ml) significantly increased cell adherence to fibronectin at 30 minutes from 4.2 ± 1.3% to 52.0 ± 4.2%, at 60 minutes from 4.8 ± 2.8% to 56.3 ± 2.8%, and at 120 minutes from 6.5 ± 4.2% to 24.5 ± 6.5%. Also, adherence to poly-L-lysine was significantly accelerated when PMNs were challenged with TNFα at 30 minutes from 29.9 ± 2.8% to 58.5 ± 8.4% and at 60 minutes from 21.8 ± 4.2% to 56.3 ± 9.8%. At 120 minutes, the effect, 23.2 ± 2.8% to 29.9 ± 8.4%, was no longer significant. Detachment of cells continued, and only 9.2 ± 3.2% of the cells were still adherent to fibronectin at eight hours. PMNs did not adhere to and did not spread with PolyHema. The PolyHema results were obtained by phase contrast microscopy of unstained PMNs, as the toluidine blue assay interfered with the PolyHema reagent.

After having demonstrated that TNFα promotes PMN adherence to fibronectin but not to PolyHema, we established the apoptosis time course in untreated and TNFα-treated PMNs on both surfaces in parallel using flow cytometry. Each time point was studied five times. The results demonstrated no significant difference in unstimulated PMNs undergoing spontaneous apoptosis up to 28 hours (percentage of apoptotic PMNs on fibronectin vs. PolyHema at eight hr, 1 ± 0.5% to 0.6 ± 0.2%; at 12 hr, 3 ± 2% to 1 ± 1%; at 18 hr, 13 ± 5% to 14 ± 6%; at 28 hr, 26 ± 6% to 34 ± 8%). However, apoptosis was accelerated in PMNs on surface-attached fibronectin compared to PolyHema, when cells were treated with 20 ng/ml TNFα. At one hour, the percentage of apoptotic cells on fibronectin and PolyHema was less than 1% for both. At two hours, these values were 9 ± 1% and 5 ± 1% ($P < 0.05$). At four hours, the difference was 19 ± 2% compared with 9 ± 1% ($P < 0.05$). At eight hours 28 ± 2% PMNs on fibronectin were apoptotic, compared with 11 ± 2% PMNs exposed to PolyHema ($P < 0.001$). At 12 hours, these values were 25 ± 2% compared with 9 ± 1% ($P < 0.001$). No significant cell loss was observed over this time period, and cell viability exceeded 95%, as determined by trypan blue exclusion (data not shown). We did a set of experiments to ensure that collecting adherent cells by scraping did not affect the flow cytometry assay for quantitation of apoptosis. After removing the nonadherent cells, apoptosis of those cells that were still adherent on fibronectin was assessed simultaneously by three different methods ($N = 2$): (*a*) collecting the cells by scraping followed by flow cytometry on ethanol fixed, PI stained cells, (*b*) staining of adherent cells directly on the dish by Wright Giemsa, and (*c*) staining of adherent cells directly on the dish by the fluorescent dye DAPI. The percentage of apoptotic cells was 28.1 ± 5.8% for PI staining; 35 ± 11.3% for Wright-Giemsa staining, and 22.8 ± 5.9% for DAPI staining. These results demonstrate that collecting adherent cells by scraping did not affect our data. We performed a DNA-fragmentation assay looking for the presence of internucleosomally cleaved DNA, a hallmark of apoptosis, as shown in Figure 1. Apoptosis, as indicated by DNA "ladders," was observed in TNFα-treated PMNs cultured eight hours on PolyHema and fibronectin, whereas no such low-molecular DNA fragments were detected in untreated controls.

Based on the results of the time course study, we selected the eight-hour time point for further experiments. Figure 2 shows the results of parallel experiments comparing apoptosis of TNFα-treated PMNs at eight hours on PolyHema, fibronectin, and poly-L-lysine ($N = 11$). A significant increase in the percentage of apoptotic cells was observed on fibronectin (37 ± 4%) compared with PolyHema (20 ± 3%) and to poly-L-lysine (25 ± 3%). Also, apoptosis of TNFα-treated PMNs on poly-L-lysine was significantly higher than on PolyHema.

To study whether other matrix proteins would acceler-

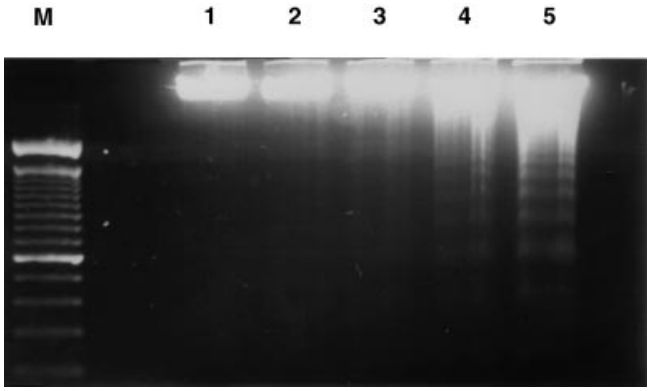

Fig. 1. Effect of fibronectin and PolyHema on DNA fragmentation of tumor necrosis factor α (TNFα)-treated polymorphonuclear neutrophils (PMNs). Cells were cultured in the absence or in the presence of 20 ng/ml TNFα for eight hours. No DNA fragmentation was observed in freshly isolated cells (lane 1) and in untreated PMNs on PolyHema (lane 2) and fibronectin (lane 3). In contrast, TNFα-treated PMNs showed low molecular weight DNA fragments on PolyHema (lane 4) and on fibronectin (lane 5). Lane M represents 100 bp DNA size markers. A typical example of two separate experiments is shown.

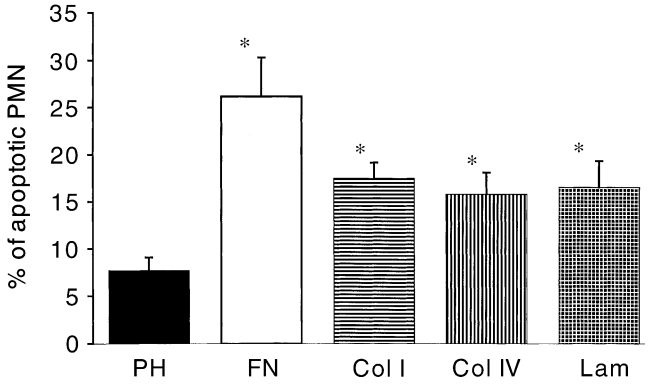

Fig. 3. Effect of different extracellular matrix (ECM) proteins on TNFα-mediated apoptosis. PMNs of the same preparation were cultured on PolyHema (PH), fibronectin (FN), collagen I (Col I), collagen IV (Col IV), and laminin (Lam). Cells were harvested after eight hours, and the percentage of apoptosis was measured using flow cytometry. Data are given as mean ± SEM ($N = 6$). *Differences between all matrices and PolyHema ($P < 0.05$) and between fibronectin and all other matrix proteins ($P < 0.05$) were significant.

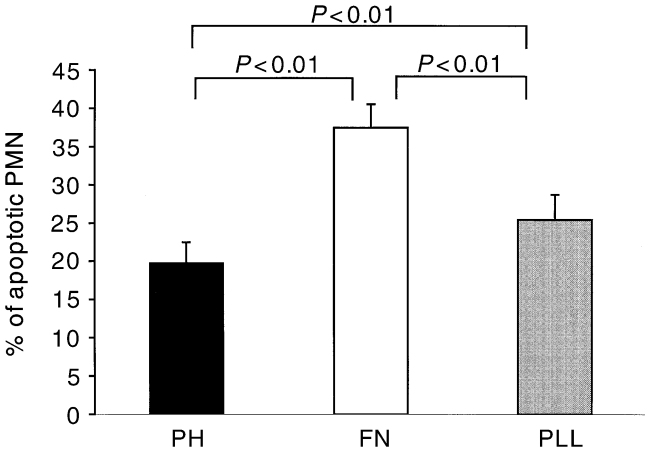

Fig. 2. Effect of PolyHema, fibronectin, and poly-L-lysine on apoptosis of TNFα-treated PMNs. Cells were treated with 20 ng/ml of TNFα for eight hours. Apoptosis was detected using flow cytometry, and data are depicted as mean ± SEM ($N = 11$). Significant acceleration of apoptosis was found in PMNs cultured on immobilized fibronectin (FN) when compared with PolyHema (PH) and with poly-L-lysine ($P < 0.01$). Also, PMN apoptosis on poly-L-lysine was significantly increased compared with PMN apoptosis with PolyHema ($P < 0.01$).

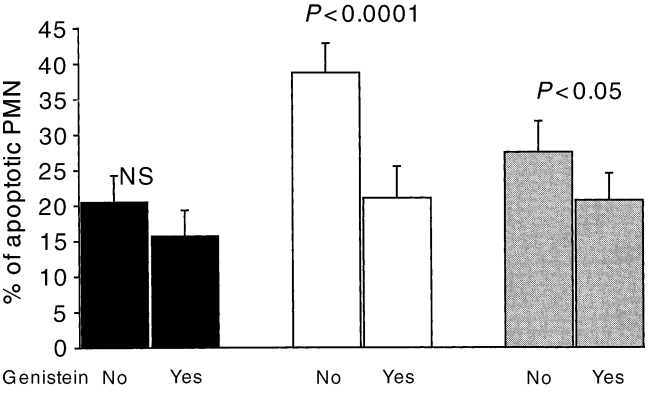

Fig. 4. Role of genistein in TNFα-mediated apoptosis. PMNs were added to wells coated with PolyHema, fibronectin, and poly-L-lysine and were preincubated with genistein (50 μM) before being stimulated with 20 ng/ml of TNFα. Apoptosis was detected after eight hours by flow cytometry. Results are expressed as mean ± SEM ($N = 8$). Significant inhibition by genistein was found in TNFα-treated cells on fibronectin (□), and poly-L-lysine (▩), but not on PolyHema (■).

ate TNFα-mediated neutrophil apoptosis, we cultured cells of the same preparation for eight hours with PolyHema, fibronectin, collagen type I and IV, and on laminin in parallel, as shown in Figure 3. The results demonstrate a significant effect of all matrix substances compared with PolyHema ($N = 6$). The percentage of apoptotic PMNs increased from 8 ± 1% on PolyHema to 26 ± 4% on fibronectin, to 17 ± 2% on collagen I, to 16 ± 2% on collagen IV, and to 17 ± 3% on laminin. The increased apoptosis on fibronectin was significantly higher than on all other matrices ($P < 0.05$).

To determine the involvement of tyrosine phosphorylation in TNFα-mediated PMN apoptosis, the effect of genistein was studied in eight independent experiments, as shown in Figure 4. The results demonstrated that genistein (50 μM) strongly inhibited TNFα-mediated apoptosis on fibronectin (39 ± 4% to 21 ± 4%; $P < 0.0001$) and on poly-L-lysine (28 ± 4% to 21 ± 4%; $P < 0.05$), whereas no significant inhibition occurred on PolyHema (20 ± 4% to 16 ± 4%). Interestingly, when TNFα-treated PMNs on both adhesive surfaces were preincubated with genistein, apoptosis was decreased to a level that was seen in nonadherent TNFα-stimulated PMNs on PolyHema.

To study potential signal transduction mechanisms

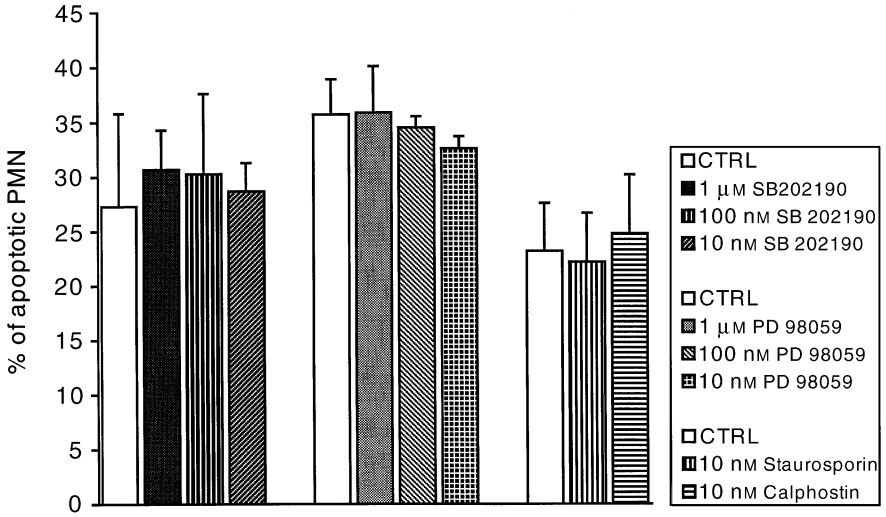

Fig. 5. Effect of serine/threonine kinase inhibitors on apoptosis of TNFα-treated PMNs (20 ng/ml) on fibronectin using flow cytometry. PMNs were preincubated with MAP-kinase inhibitors SB 202190 and PD 98059 (10 nM to 1 μM) or with calphostin and staurosporin (10 nM) to inhibit PKC. Results are means ± SEM ($N = 4$ for SB 202190 and $N = 3$ for all other experiments).

further, we tested the effect of the MAP-kinase inhibitors SB 202190 and PD 98059 (10 nM to 1 μM), as well as the effect of the PKC inhibitors calphostin and staurosporin (10 nM). At these concentrations, neither of these inhibitors affected TNFα-mediated neutrophil apoptosis on fibronectin, as shown in Figure 5.

Because genistein inhibited TNFα-mediated apoptosis on fibronectin, we studied tyrosine phosphorylation in more detail. We used PMN lysates of cells that were cultured in the absence or in the presence of TNFα, on either PolyHema or on fibronectin. We performed SDS-polyacrylamide gel and Western blotting with an anti-phosphotyrosine antibody, as shown in Figure 6. Cells were incubated for 15, 30, or 60 minutes, respectively. At 15 minutes, no significant difference was observed, whereas after 30 minutes, an increased tyrosine phosphorylation of a protein band with a molecular weight of 95 and 54 kDa was observed in TNFα-treated cells on fibronectin. The phosphorylation of these proteins increased further at 60 minutes, together with proteins at 102, 89, 63, and 51 kDa. Interestingly, a number of newly phosphorylated proteins were observed with an apparent molecular weight of 185, 85, 66, 56, and 42 kDa.

We then used confocal microscopy to confirm the fibronectin-induced tyrosine phosphorylation in PMNs and to localize phosphorylated proteins. Tyrosine phosphorylation was assessed in immunostained PMNs on fibronectin or with PolyHema. As shown in the panels of Figure 7, we observed a marked increase in tyrosine phosphorylation at 60 minutes when TNFα-treated PMNs were cultured on fibronectin compared with cultures with PolyHema (Fig. 7, A compared with B, as quantitated in C). PMN adherence to fibronectin was associated with a change in cell shape (Fig. 7D). The increased tyrosine phosphorylation was localized to cellular processes mediating cell-matrix contact. The vertical analysis (z-axis) of the fibronectin-adhered cells also showed an increase in tyrosine phosphorylation at the cell borders and in the cytoplasm (Fig. 7E).

During our experiments, we observed an inhibitory effect of genistein on cell spreading. In a final set of experiments, we further investigated this issue. We studied the effect of TNFα and the effect of genistein pretreatment on spreading of PMNs cultured on fibronectin and on poly-L-lysine in parallel ($N = 7$). Cell spreading was observed only when cells were treated with TNFα (20 ng/ml), and the percentage of spread cells was higher on fibronectin than on poly-L-lysine (at 30 min, 51% to 17%, $P < 0.05$; 60 min, 77% to 27%, $P < 0.05$; and 120 min, 29% to 14%, NS). In contrast, when PMNs were preincubated with 50 μM genistein prior to the treatment with TNFα, cell spreading on either fibronectin or poly-L-lysine was decreased to less than 3% at all three time points. In addition, genistein also decreased cell adhesion on fibronectin (85.7% at 30 min, $P < 0.05$; 52.4% at 60 min, $P < 0.001$; 47.6% at 120 min, $P < 0.01$) and on poly-L-lysine (90.0% at 30 min, $P < 0.05$; 74.6% at 60 min, $P < 0.001$; 86.3% at 120 min, NS).

DISCUSSION

We observed that the interaction between PMN and ECM proteins in the presence of the proinflammatory cytokine TNFα results in accelerated apoptosis, whereas constitutive apoptosis was not affected. Studies on signal transduction pathways revealed that accelerated apoptosis in TNFα-treated PMNs was diminished by inhibiting tyrosine phosphorylation, whereas no significant effect was seen on a surface that prevented cell adhesion. By Western blot analysis, we observed increased tyrosine phosphorylation of discrete bands and the appearance of newly phosphorylated proteins only in TNFα-treated

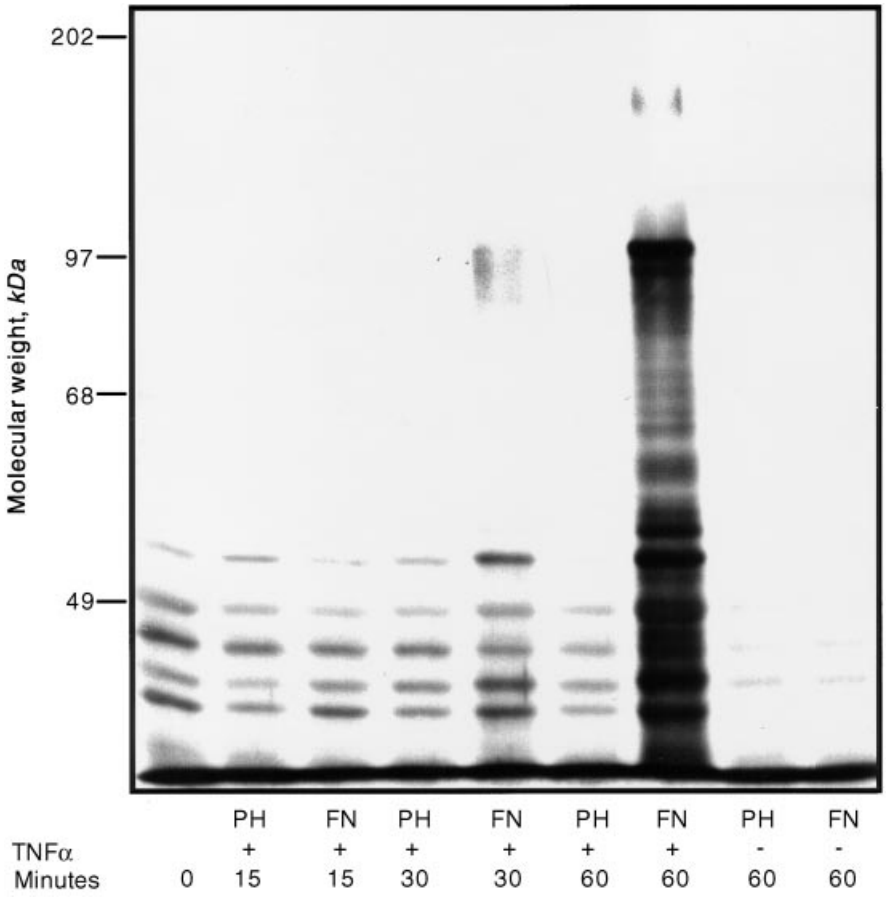

Fig. 6. Detection of tyrosine phosphorylation by Western blotting. PMNs were cultured on fibronectin or on PolyHema and either stimulated with 40 ng/ml of TNFα or left untreated. Samples were harvested after 15 minutes, 30 minutes, and 60 minutes 50 μg of protein/lane were electrophoresed, blotted onto a membrane, and probed using the monoclonal antibody PY20. Tyrosine phosphorylation was only up-regulated in TNFα-treated PMNs on fibronectin. Tyrosine phosphorylation was increased in protein bands of approximately 102, 95, 89, 63, 54, and 51 kDa. New phosphorylated protein bands were visible at approximately 185, 85, 66, 56, and 42 kDa. A typical example of three separate experiments is shown.

PMNs on fibronectin. Confocal microscopy demonstrated high tyrosine phosphorylation in focal adhesion sites and in the cytoplasm of TNFα-treated PMNs on fibronectin.

Approximately 10^{11} PMNs are released from the bone marrow daily [22]. Acute tissue inflammation, including glomerulonephritis, results in recruitment and local activation of PMNs. To accumulate at the inflamed site, PMNs transmigrate through the endothelial interface into tissue interacting with ECM substances in the presence of inflammatory mediators, including cytokines. PMN responses, such as respiratory burst, degranulation, and phagocytosis, are adhesion dependent [4, 5, 7, 8, 23]. However, tissue inflammation is usually self-limiting. Studies have indicated that PMNs undergo apoptosis at inflammatory sites *in vivo* and that this mechanism participates in the resolution of inflammation [10, 24]. Apoptotic PMNs have a decreased biological response *in vitro* [13, 25, 26] and are recognized by macrophages, even without neutrophil disintegration or macrophage activation [24]. PMN apoptosis is relevant to consecutive cell clearance *in vivo*, as shown in various diseases, such as pulmonary inflammation, rheumatoid arthritis, and glomerulonephritis [1, 27].

Binding to ECM promotes epithelial and endothelial cell survival [28, 29]. The loss of contact with the underlying matrix, termed anoikis, results in cell apoptosis. Integrins are involved in this anchorage-dependent cell survival [30, 31]. However, PMNs arrive either as a pool of nonadherent cells when circulating in the blood stream or interact with the endothelial interface and ECM when migrating into tissue. Our understanding of how these different conditions affect PMN apoptosis is largely incomplete. Very recent studies demonstrated that PMN apoptosis is modulated by adhesion to endothelial cells [32], by transendothelial transmigration [33], and by platelets [34]. A role for integrins and selectins has been suggested under some conditions [35, 36]. However, the significance of ECM for neutrophil survival is less clear. Our data indicate that apoptosis is accelerated in PMNs interacting with ECM in the presence of the proinflammatory cytokine TNFα. This finding contrasts with the delay of apoptosis seen when unstimulated endothelial cells bind to ECM. Apoptosis acceleration was observed only when the cells interacted with immobilized matrix protein because the addition of soluble fibronectin to cells in suspension had no effect. Spontaneous apoptosis was not affected during the observation period.

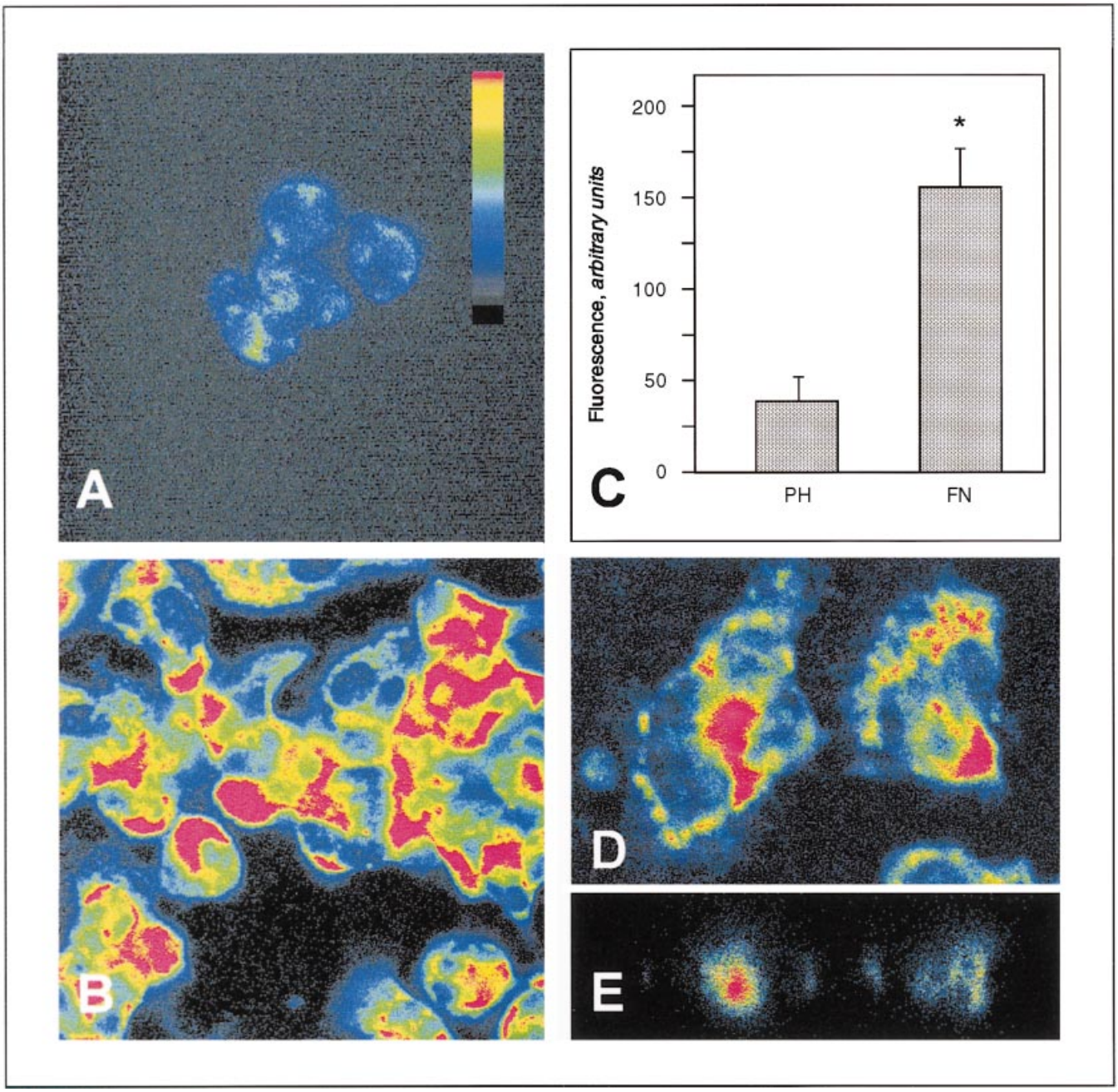

Fig. 7. Confocal microscopy of tyrosine phosphorylation in TNFα-treated PMNs with PolyHema (*A*) and on fibronectin (*B*). A strong up-regulation of phosphotyrosine was detected in TNFα-treated PMNs on fibronectin (B). Semiquantitative analysis is shown in (*C*). Note that cells on fibronectin were well spread with increased tyrosine phosphorylation at sites of cell–matrix contact and in the cytosol (*D*, horizontal section). In (*E*), a vertical analysis (z-axis) of a fibronectin-adhered cell also showed an increase in tyrosine phosphorylation at the cell borders and in the cytoplasm (E). The 60-minute time point of a representative example from three experiments is shown.

The signal transduction of PMN apoptosis is largely unknown. A critical role for PKC [37] and protein tyrosine phosphorylation [38, 39] was demonstrated by the inhibition of PMN apoptosis by granulocyte-macrophage colony-stimulating factor. Furthermore, PMN apoptosis induced by ligation of Fas was suppressed by tyrosine kinase inhibitors [40]. The MAP kinase cascade was shown to be a key element in TNFα-induced apoptosis in COS cells [41]. In fact, also PMNs stimulated with TNFα activate p38 and p42 MAP kinase [42, 43]. Our data indicate the importance of tyrosine phosphorylation for the fibronectin-mediated acceleration of apoptosis in TNFα-treated PMNs. Neither the PKC nor the MAP kinase pathway seemed to play a role in this condition.

However, we performed no specific experiments to demonstrate for certainty that the concentrations we employed inhibit these enzymes. We showed that acceleration of apoptosis on ECM was associated with increased tyrosine phosphorylation of several proteins and with the appearance of newly tyrosine phosphorylated proteins. We do not know the nature of these phosphoproteins. However, based on their molecular weights, several assumptions can be made. Tyrosine phosphorylated proteins of 56 kDa and 54 kDa could represent members of the *src* kinase family such as *lyn* and *fgr.* A role of lyn-protein tyrosine kinase in PMN apoptosis has been suggested by others [39]. The 42 kDa protein could conceivably be MAP kinase. However, our negative results using specific inhibitors of this kinase would rule out a role of MAP kinase p42 in the mediation of PMN apoptosis under these conditions.

Using confocal microscopy, we found increased tyrosine phosphorylation at contact sites between adherent PMNs and ECM. Interestingly, in this study, acceleration of apoptosis as well as cell spreading was prevented by genistein. This finding implies that cell spreading may be one of the important factors for propagating PMN apoptosis. Cell spreading is important for several other neutrophil functions, including the respiratory burst, a process that is regulated by protein tyrosine phosphorylation [44, 45]. Members of the *src* family kinases p59/61^{hck} and p58c^{fgr} were shown to be important substrates [45]. The role of cell spreading for the acceleration of PMN apoptosis on ECM is interesting. This observation should be diligently explored in future studies.

In summary, our findings indicate that the interaction between PMNs and ECM results in accelerated apoptosis when the proinflammatory cytokine TNFα is present. This process is controlled by tyrosine phosphorylation. The increase in protein tyrosine phosphorylation in TNFα-treated PMNs on fibronectin occurs most likely in response to both the effect of TNFα and the effect of adherence and spreading. The matrix effect may participate in the local control of inflammation when PMNs leave the circulation and migrate into inflamed tissue.

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APPENDIX

Abbreviations used in this article are: BSA, bovine serum albumin; DAPI, 4,6 diamidino-2-henylindole; ECM, extracellular matrix; EDTA, ethylenediaminetetraacetic acid; MAP, mitogen-activating protein; OD, optical density; PBS, phosphate-buffered saline; PI, propidium iodide; PKC, protein kinase C; PMNs, polymorphonuclear neutrophils; PolyHema, poly-hydroxyl-ethyl-meth-acrylate; SDS, sodium dodecyl sulfate; TNFα, tumor necrosis factor-α.

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